Microfluidic patterning of nanodisc lipid bilayers and multiplexed analysis of protein interaction†

Edgar D. Goluch,‡a Andrew W. Shaw,‡b Stephen G. Sligarb,c,d and Chang Liu*a,e,f

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We report a microfluidic method for precisely patterning lipid bilayers and a multiplexed assay to examine the interaction between the lipids and protein analytes. The lipids were packaged into nanoscale lipid bilayer particles known as Nanodiscs and delivered to surfaces using microfluidic channels. Two types of lipids were used in this study: biotinylated lipids and phosphoserine lipids. The deposition of biotinylated lipids on a glass surface was confirmed by attaching streptavidin coated quantum dots to the lipids, followed by fluorescent imaging. Using this multiplexed grid assay, we examined binding of annexin to phosphoserine lipids, and compared these results to similar analysis performed by surface plasmon resonance.

Introduction

The cellular membrane is the location of many biochemically and medically important processes. Ion transport, hormone signaling, blood coagulation, and endocytosis are just a few examples of the numerous important cellular processes that occur at the lipid bilayer membrane. It is therefore of great interest to develop high-throughput screening techniques involving lipid bilayers and membrane-associated proteins.

Microfluidic devices are attractive options for this screening task. A number of high-throughput assays, including DNA analysis,¹–³ protein analysis,⁴,⁵ and immunoassays,⁶,⁷ have already been demonstrated with this technology. Here, we report a new strategy for producing precise patterns of lipid bilayers with stoichiometric control of the bilayer composition. Specifically, a microfluidic device is employed to deliver nanoscale lipid bilayer constructs, known as Nanodiscs,⁸,⁹ to the desired surface. This approach is further utilized to examine the properties of interactions between surface immobilized lipids and proteins in solution.

Several methods have been employed to deposit spatially resolved lipid bilayers, including patterning with microfluidic channels. However, these methods primarily used lipid aggregates known as liposomes, and did not demonstrate arrays of lipids with varying compositions.¹⁰–¹⁸ Liposomes are tens of nanometres to microns in diameter. Upon interaction with surfaces, they exhibit instability and undergo fusion and aggregation. Furthermore, the size of liposomes is many times larger than that of proteins, preventing stoichiometric control of membrane receptors and limiting the density at which they can be packed on a surface.

To overcome the difficulties associated with liposomes, Nanodiscs are employed to deliver lipids to the surface. Unlike liposomes, Nanodiscs provide a completely soluble nanoscale section of lipid bilayer, which can be handled in the same manner as other biological macromolecules. Further, they can be assembled with stoichiometric control over the number of membrane receptors incorporated into each Nanodisc.¹⁹–²¹ These robust structures are formed through a self-assembly reaction between detergent solubilized lipids and an amphipathic helical protein called membrane scaffold protein (MSP) designed for disc formation, as shown in Fig. 1. Removal of the detergent initiates the self-assembly reaction, which yields a disc-shaped

Fig. 1 (A) Formation of Nanodiscs occurs by mixing membrane scaffold protein with detergent solubilized phospholipids (B) followed by removal of the detergent. (C) The result is a discoidal phospholipid bilayer encircled by protein measuring 9.5–10 nm in diameter.
lipid bilayer encircled by two MSPs with an overall diameter of approximately 10 nm.

Microfluidic channels are formed by placing molded poly(dimethylsiloxane) (PDMS) chips on standard glass slides (Fig. 2). No special glass preparation is required as Nanodiscs are known to physisorb directly to glass surfaces in the same way as other lipid bilayers.\textsuperscript{8,22,23} Solutions containing Nanodiscs are flowed through the channels. The channels are emptied before the PDMS chip is removed from the surface, thus creating a moderately dry patterned surface. This step precisely deposits Nanodiscs according to the channel geometries. Atomic force microscope (AFM) analysis, by Bayburt et al., of Nanodiscs deposited on mica using similar concentrations has shown that a tightly bound monolayer of intact discs is formed on the surface with one face of the lipid bilayer toward the mica surface and the other exposed to the solution.\textsuperscript{8}

Fig. 2  Schematic of microfluidic grid assay. (A) First, a PDMS chip is flowed on a microscope slide. (B) Solutions containing Nanodiscs are flowed through the channels. (C) The PDMS chip is removed. (D) A second PDMS chip is placed on the substrate with channels perpendicular to the first chip. (E) Solutions containing binding targets are flowed through the channels. (F) The chip is removed and the slide is scanned to visualize the results.

A second PDMS chip is then placed over the patterned area, with the direction of its channels perpendicular to the earlier case. Analyte solutions are then delivered across the rows of patterned Nanodiscs, creating a two dimensional array of interaction sites to investigate Nanodisc–analyte interactions in a multiplexed manner. The assay presented here is performed on a standard glass slide, uses sample volumes of 5 to 10 \( \mu \)L, and takes approximately 30 min to complete.

In order to demonstrate the immobilization of lipids and binding of protein analytes, we used two systems. The first system employed Nanodiscs containing biotinylated lipids. The delivery of biotinylated lipids was detected using streptavidin-labeled quantum dots. Biotin–streptavidin provides an extremely strong and well-characterized protein-target binding system that is ideal for demonstrating the feasibility of this technique.\textsuperscript{24,25}

The second system involves Nanodiscs containing various amounts of phosphoserine (PS) lipids. These Nanodiscs are screened with annexin, a family of proteins that play a significant role in various cellular functions.\textsuperscript{26–28} Annexin assays are widely used to determine apoptosis, an important biological process that malfunctions in many diseases such as cancer and Alzheimer’s. Specifically, this work utilized annexin V, a protein involved in endocytosis which binds to negatively charged PS lipids.\textsuperscript{29} The binding events are monitored by employing fluorescently labeled annexin. A similar experiment was carried out using surface plasmon resonance (SPR), a leading technique for analysis of protein binding due to its ability to monitor binding in real-time without the use of labeled molecules.\textsuperscript{30–32}

Materials and methods

Fabrication of microchannels

Positive photoresist (AZ4620, Clariant, Somerville, NJ, USA) was spin-coated (2200 rpm, 60 s) on a 75 mm diameter Pyrex glass flat (Esco, Oak Ridge, NJ, USA) and baked at 110\( ^\circ \)C for 2 min. The photoresist was patterned using standard photolithography techniques to yield 12 \( \mu \)m tall features. The patterned wafers were then exposed to chlorotrimethylsilane (Dow Corning, Midland, MI, USA) vapor for 1 min to facilitate release of PDMS molds from the substrate.

A 10 : 1 v/v mixture of PDMS prepolymer and curing agent (Sylgard 184, Dow Corning, Midland, MI, USA) was prepared, degassed, and poured on the patterned glass flat. The PDMS was cured at 90 \( ^\circ \)C for 2 h, removed from the substrate, and baked overnight at 90 \( ^\circ \)C to fully cure the chip. Inlet and outlet holes were drilled in the PDMS using a sharpened 18-gauge needle. Final dimensions of individual channels were 200 \( \mu \)m wide, 12 \( \mu \)m tall, and 2 cm long.

Nanodisc production

A detailed discussion on the method of Nanodisc production and characterization has been previously published.\textsuperscript{8} Herein, we provide a brief summary of the process. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine] (POPS), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and \( N \)-(biotinyl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (biotin-DPPE) were purchased from Avanti Polarlipids Inc. (Alabaster, AL, USA). Stock solutions of each phospholipid were made in chloroform with concentrations determined by inorganic phosphate analysis. Solutions were stored at \( -20 ^\circ \)C. The membrane scaffold protein MSP1T2 was expressed in \textit{E. coli} with an oligohistidine tag and purified using nickel affinity chromatography as previously described.\textsuperscript{8} Aliquots of lipid stock solutions were mixed in glass test tubes to achieve the composition of lipids desired in Nanodiscs (DPPC with or without biotin-DPPE and POPC with various amounts of POPS). Excess chloroform was blown off with nitrogen, and lipids were placed under vacuum overnight to ensure complete removal of the solvent. Lipids were resuspended by sonication with heating and vortexing in an aqueous solution of sodium cholate added to give a 2 : 1 molar ratio of cholate : lipid. MSP was added to give a lipid : MSP molar ratio of 70 : 1 for unsaturated lipids (POPC and POPS) and 90 : 1 for saturated lipids (DPPC and biotin–DPPE). The mixture was incubated for 1 h at approximately the phase transition temperature of the lipid used (4 \( ^\circ \)C for POPC and POPS and 37 \( ^\circ \)C for DPPC and biotin–DPPE). Bio Beads (Biorad Corp.) were then added in the amount of 400 mg per millilitre of volume to facilitate detergent removal. The mixtures
were then incubated again at the same temperatures as the first incubation for 3–4 h with shaking. The solution was separated from the Bio Beads and run on a Sephadex size exclusion to remove any aggregates and confirm disc formation. The resulting peak confirms monodisperse particles with a diameter of 10 nm. Quantitation of lipids using inorganic phosphate analysis has shown Nanodiscs to contain approximately 160 saturated lipids or 130 unsaturated lipids. Nanodisc concentration was determined by absorbance of the MSP at 280 nm (extinction coefficient = 21 000 M⁻¹ cm⁻¹).

Preparation of substrates

Microscope slides (Fisher, Hanover Park, IL, USA) were cleaned using 100% ethanol followed by thorough rinsing with 18.2 MΩ deionized water (Millipore Synthesis, Millipore, Billerica, MA, USA). They were then dried under a stream of nitrogen.

Assay protocol

The PDMS piece was sealed against the glass substrate to form enclosed channels. Conformal contact is achieved spontaneously through Van der Waals interactions between the two materials. After the channels were formed, solutions were pulled through the microfluidic channels using a syringe pump (74900, Cole-Parmer, Vernon Hills, IL, USA) at a flow rate of 1 μL min⁻¹.

First, 10 μL of 100 nM Nanodisc solution (in 10 mM Tris, pH = 7.4, 150 mM NaCl) was flowed through the channels to pattern the surface. Then, the channels were washed with 5 μL of buffer (10 mM Tris, pH = 7.4, 150 mM NaCl). If whole channel assays were performed, the PDMS chip was left in place and the analyte was flowed through the same channels. For grid assays, the PDMS chip was removed and a second PDMS chip was attached with channels perpendicular to the original pattern. 5 μL of 1% BSA (Invitrogen, Carlsbad, CA, USA) was flowed through the channels to prevent non-specific adsorption of the targets. Next, 5 μL of the binding target, either streptavidin coated quantum dots (565 nm for whole channel assay, 605 nm for the grid assay, Invitrogen, Carlsbad, CA, USA) or Cy5 conjugated annexin (A35108, Invitrogen, Carlsbad, CA, USA) (in 10 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM CaCl₂) was flowed through the channels. Unbound target was removed by flowing 5 μL of buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM CaCl₂) through the channels. The PDMS chip was removed from the glass, which was then rinsed thoroughly with agitation for 1 min in buffer. Finally, the glass was spin-dried for 10 s at 6000 rpm using a high-speed minicentrifuge (ArrayIt, Sunnyvale, CA, USA).

Fluorescence imaging

Glass slides were visualized using an Axon 4200A GenePix Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA) using a 488 nm wavelength laser for the quantum dot system and 532 nm laser for the Cy5 conjugated annexin. The photomultiplier tube (PMT) level was set at 450 for all experiments. Fluorescent images were processed using GenePix Pro 6.0.

Surface plasmon resonance (SPR)

Binding of annexin (A9460, Sigma-Aldrich, St. Louis, MO, USA) to Nanodiscs containing POPC and POPS was quantified by SPR using a Biacore 3000 instrument (Biacore Inc., Piscataway, NJ, USA). Nanodiscs were attached to an NTA (nitrotritriacetic acid) Biacore sensor chip in a manner similar to that used in Shaw, et al. Briefly, the sensor chip was charged with nickel using the method recommended by the manufacturer, and Nanodisc immobilization was achieved via the oligohistidine tag of the membrane scaffold protein. The running buffer consisted of 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) pH 7.4 adjusted with NaOH, 150 mM NaCl, 2.5 mM CaCl₂, flowed at 5 μL min⁻¹. 250 resonance units of Nanodiscs of various PS content were loaded onto channels in calcium-free running buffer after which stable baselines were established in running buffer. Kinetic injections of annexin were run in parallel on the Nanodiscs. A channel loaded with POPC Nanodiscs was used to subtract changes in response units (RU) due to refractive index changes of the protein solutions and any non-specific binding to the channels. The NTA chip was regenerated using 0.35 M ethylene diamine tetraacetic acid (EDTA) buffer, which successfully removed the Nanodiscs and any bound annexin.

Results and discussion

To demonstrate the ability to immobilize lipids on a surface, Nanodiscs containing biotinylated lipid were patterned using microfluidic channels and challenged with streptavidin-coated quantum dots (streptavidin–QDs) flowed through the same channels. We prepared a chip containing three channels representing three cases (Fig. 3). Nanodiscs containing DPPC : biotin-DPPE (9 : 1 mole ratio) were flowed through channels A and B. In channel C, DPPC Nanodiscs containing no biotinylates were used as a control. After the channels were washed with running buffer and subjected to the BSA solution, the PDMS chip was left in place instead of being removed. Solutions containing streptavidin–QDs were flowed through the channels, with the streptavidin–QD concentrations being 10 nM for channel A, 1 nM for channel B, and 10 nM for channel C. Following a final wash with buffer, the slide was spin-dried and imaged.

![Fig. 3](image-url) Fluorescence image of Nanodiscs patterned using microchannels followed by exposure to streptavidin-coated quantum dots (585 nm). (A) Biotin–DPPE Nanodiscs exposed to 10 nM streptavidin-coated quantum dots. (B) biotin–DPPE Nanodiscs exposed to 1 nM streptavidin-coated quantum dots, and (C) DPPC Nanodiscs exposed to 10 nM streptavidin-coated quantum dots.

As can be seen, the two channels in which biotinylated Nanodiscs were patterned (A and B) exhibit high fluorescence intensity while the channel in which Nanodiscs without biotin were patterned (C) shows no more fluorescence than the background. This experiment yields many important performance characteristics of the assay. First, it demonstrates the selective binding of the streptavidin–QDs to the biotin target present...
at the bilayer of the Nanodiscs. Second, the fluorescence intensity is constant throughout the channels containing biotin–Nanodiscs, showing a homogeneous patterning of the discs throughout the length of the channel. Third, the lipids are not removed by repeated washing procedures. Finally, of the two channels with biotinylated Nanodiscs, the one subjected to a higher concentration of streptavidin–QDs (A), yields a stronger fluorescence signal, suggesting the assay can deliver quantitative results.

The experiment was repeated using the grid assay method illustrated in Fig. 2. We patterned six rows of Nanodiscs, with biotin–Nanodisc concentrations of 100, 75, 50, 25, and 10 nM as well as a control row with 100 nM DPPC Nanodiscs containing no biotinylated lipids. Streptavidin solutions with six different concentrations (5.0 nM, 2.5 nM, 1.25 nM, 0.6 nM, 0.3 nM, blank) were flowed through six cross channels, forming a grid of $6 \times 6$ reaction sites. The experiment was repeated four times to ensure reproducibility. The fluorescence image of a typical assay is shown in Fig. 4. The fluorescence intensity generally increases with either increasing concentration of Nanodiscs or streptavidin–QD solutions. For each Nanodisc concentration, the signal intensity increases monotonously with increasing concentrations of quantum dots. For each quantum dot concentration, the fluorescent signal intensity increases between Nanodisc concentrations of 10 and 50 nM. Beyond 50 nM, the signal intensity saturates. This experiment proves that the assay provides quantifiable data for a decade of Nanodisc concentrations and a decade of quantum dot dilutions.

A Nanodisc concentration of 50 nM represents the upper limit of surface coverage in the microfluidic channels. Experiments carried out at higher Nanodisc concentrations do not show any further increase in fluorescence. The lower detection limit is restricted by the sensitivity of the imaging instrument. More sensitive fluorescence detection techniques are available, but they currently lack the large field of view necessary for this experiment.

To further investigate protein interaction with the lipid bilayer, we studied annexin binding to Nanodiscs containing PS compositions ranging from 0–100%. The lipid composition in Nanodiscs made with POPC and POPS has been shown to be identical to the ratio of the lipids in the starting mixture through analysis of radiolabeled lipids. It is important to note that liposomes containing high amounts of PS are not stable in the presence of calcium, which is necessary for binding the annexin. The results in Fig. 5 show that the amount of annexin binding increases with increasing amounts of PS incorporated into the Nanodiscs. Also, the signal decreases as the annexin concentration in solution is lowered. The grid was reproduced six times to validate the results. Saturation of annexin binding occurs for Nanodiscs containing 60%, 80%, and 100% PS and at high annexin concentrations (dilutions less than 25 times).

The regular vertical line structures on the left side of Fig. 5A are observed when high concentrations of proteins are flowed through the channels. Small amounts of fluid remain in the corners of the microfluidic channels, even after the channel is emptied, due to no-slip boundary conditions at the channel wall. This fluid evaporates almost instantly when the PDMS chip is removed, leaving behind proteins on the surface. However, this does not affect the results of the experiments. The fluorescent readings are taken only at the channel intersections and in an area slightly smaller than the channels themselves, thereby avoiding signals generated by non-specific adsorption.

We performed experiments at multiple flow rates and analyze concentrations to ensure accurate results and to identify the effects of mass transport on the binding events. A whole channel assay was run in which the annexin flow rate was varied significantly for four different concentrations. The Nanodisc PS concentration for all of the flow rate experiments was kept constant at 60%. If equilibrium were not established, one would expect to see a decrease in signal with increasing flow rate, as fewer target molecules would have time to attach to the chip surface for a constant fluid volume.

Fig. 6 shows the signal intensity generated by four different flow rates at three annexin concentrations with a constant sample volume of 5 µL. The signal was analyzed at 5 different points along the channel to monitor annexin depletion from the
Fig. 5 (A) Fluorescence image of a Nanodisc–annexin 6 x 6 microfluidic matrix. The horizontal channels contained, from top to bottom, Nanodiscs with PS ranging from 100% to 0%. Vertical channels contained fluorescent Cy5–Annexin at various dilutions. Scale bar is 200 μm. (B) The graph shows the fluorescent response for various combinations of PS Nanodiscs and annexin dilutions averaged over six experiments (error bars represent the standard deviation for 6 experiments).

bulk solution. Small error bars indicate that the signal intensity is uniform across the length of the channel. A sample volume of 5 μL is sufficient to establish equilibrium conditions throughout the length of the channel. The relatively uniform signal among the flow rates for a given concentration indicates that the signal is independent of the flow rate. The fluorescence intensity is therefore only dependent on the concentrations present in the system.

The annexin binding results from the grid assay were validated using SPR. Stable baselines were achieved reproducibly after the binding of 250 resonance units of Nanodiscs to an NTA sensor chip. This demonstrates that Nanodiscs allow for the attachment of lipid bilayers to sensor chips via the oligohistidine tag of the MSPs, and further shows that defined amounts of a lipid bilayer can be loaded onto the chip, which is necessary to perform kinetic studies in binding experiments. The maximum responses of annexin association from three minute kinetic injections are plotted in Fig. 7. The signal intensity trend matches that of the microfluidic grid assay, with increasing annexin concentrations and PS levels generally resulting in increased signal. Binding greatly increases at all concentrations of annexin between 20% and 40% PS Nanodiscs. This is similar to the increase observed between 40% and 60% PS Nanodiscs in the fluorescence-based grid assay (Fig. 5). The difference in PS is likely due to the overall higher concentrations of annexin needed for the SPR experiments.

Fig. 6 Graph of the fluorescence intensity obtained by binding annexin at various concentrations (25x, 50x, 100x) to 100 nM Nanodiscs with 60% PS at different flow rates (1 μL min⁻¹, 2 μL min⁻¹, 3 μL min⁻¹, 3.5 μL min⁻¹). Error bars are generated by evaluating the signal at 5 separate points along the channel.

Fig. 7 Surface plasmon resonance results for annexin binding to Nanodiscs of various PS compositions.

Conclusions

This experiment shows, for the first time, the feasibility of producing heterogeneous, predefined patterns of lipid bilayers using Nanodiscs as the delivery system. Regions with distinctly different lipid compositions were easily and quickly created on a glass surface. Using microfluidics, we have demonstrated a simple approach for transporting, patterning, and assaying the lipids. This method allows for the creation of high density, parallel assays for biomolecules that normally require special handling. The work presented here demonstrates the use of Nanodiscs for creating functional surface-based arrays of
membrane proteins, an important class of biomolecules for which high-throughput analysis is extremely desirable.

The Nanodisc system allows for the handling of lipid bilayers as soluble molecules, which can now be incorporated into traditional and novel biological assays. Further, we were able to analyze the binding of proteins with these lipid bilayers in a highly parallel manner using a grid array assay. The assay uses minute sample volumes, can be run quickly, and does not require expensive equipment.

It is possible to increase the matrix dimensions to hundreds of channels. The limiting factor in scaling up this technique lies in the number of pipetting steps that are required for running different solutions through each channel. It may be possible to introduce concentration gradients within the microfluidic channels in order to reduce the number of pipetting steps. Future work will include the detection of protein and small molecule interactions with membrane proteins incorporated into Nanodiscs. On-chip dilutions of analytes will result in rapid determination of binding isotherms. This ability will allow for studies in membrane protein functionality and high-throughput screening and development of new pharmaceutical drugs.

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